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Highly Potent 1-Aminocyclohexane-1-Carboxylic Acid Substituted V_2 Agonists of Arginine Vasopressin

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The synthesis and some pharmacological properties of two sets of analogues, one consisting of six peptides with 1-aminocyclohexane-1-carboxylic acid (Acc) in position 2 and the other with the amino acid in position 3, have been described. All the peptides were tested for their pressor, antidiuretic, and uterotonic in vitro activities. The Acc^2 modification has been shown to selectively modulate the activities of the analogues. Four of the compounds were highly potent antidiuretic agonists with different pressor and uterotonic activities. On the other hand, the 3-substituted counterparts failed to exhibit any of the activities. One exception was provided by the [Mpa^1,Acc^3,Val^4,D-Arg^8]VP analogue, which exhibited antidiuretic activity matching that of AVP, yet, unlike AVP, it was fairly selective.

Introduction

The synthesis of arginine vasopressin (AVP) in the early 1950s stimulated not only the fields of synthetic chemistry and peptide endocrinology but also the structure−activity relationship studies of this hormone and particularly the search for analogues with both high and specific activities. Investigations undertaken in many laboratories resulted in a number of potent and fairly selective agonists and antagonists of this peptide. However, the design of compounds that are active, truly selective, and useful as drugs continues to be a challenge.[1−3]

It is believed that the tyrosine residue in position 2 of AVP is involved in initiating the pressor response to AVP, while phenylalanine in position 3 seems to play a role in recognition of this hormone.[4] Early findings demonstrating that aromatic Phe^3 is important for vasopressor activity, whereas aliphatic Ile^3 is crucial for oxytocic activity, probably influenced the design of neurohypophyseal hormone analogues because initially only a few studies were undertaken that involved substitutions in position 3 of AVP. More recent reports on such analogues resulted in inconsistent information, on one hand supporting the conclusion that this position is intolerant to structural modifications[5−8] and on the other hand demonstrating that Phe^3 could be replaced by a variety of amino acids with appreciable retention of activity.[7−9] In 1997 we described AVP analogues modified in position 3 with β-(1-naphthyl)-L-alanine. The results suggested that position 3 was important not only for binding and recognition but also for intrinsic activity.[10] As regards position 2 of AVP, it has been shown that single substitution yields analogues with very interesting pharmacological properties.[1,3,11] However, only a limited number of 2-substituted peptides have been reported. Most of the substitutions consist of replacements with aromatic amino acid residues and their derivatives. In contrast, the influence of various modifications of position 2 on pharmacological properties is, in the case of AVP antagonists, well explored.[1,3,11]

Bearing all this in mind, we recently replaced the residues in position 2 or 3 of AVP and some of its agonistic and antagonistic analogues with 1-aminocyclohexane-1-carboxylic acid (Acc).[12] Acc was chosen to reduce the flexibility of peptides by implanting a sterically constrained residue, thus forcing the peptide backbone and side chains to adopt specific orientations.[12,13] The Acc^3 modification has been found to be deleterious for interaction with all three neurohypopheseal hormone receptors, as judged by the several orders of magnitude decreased biological activities, whereas Acc^2 substitution selectively altered the interaction with the receptors. Two of the new analogues [Acc^2]AVP and [Acc^2,D-Arg^8]VP turned out to be potent antidiuretic agonists. [Acc^2]AVP exhibited moderate pressor agonistic activity and weak antiuterotonic properties. [Acc^2,D-Arg^8]VP has been found to be a weak antagonist in pressor and uterotonic tests. Moreover, it was interesting to note that one of the Acc^3 substituted peptides, namely, [Cpa^1,Acc^3]AVP, turned out to be a selective V_2 agonist.

These, in our opinion, are very interesting results and prompted us to further investigate the influence of the Acc^2 and Acc^3 modifications on pharmacological properties of agonistic and antagonistic analogues of AVP. It is well-known that deamination is the most effective of the individual changes in AVP that lead to enhanced antidiuretic activity, whereas inversion of configuration of arginine in position 8 results in analogues with distinctly increased specificity of antidiuretic action.[2,11] On the other hand, valine in position 4 of the AVP analogues improves, in many cases, antidiuretic potency and selectivity.[2,11] These facts prompted us to synthesize and evaluate biological activities of the following analogues: [Mpa^1,Acc^2]AVP (**I**), [Acc^2,Val^4]AVP (**II**), [Cpa^1,-

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Table 1. Physicochemical Properties of Peptides **I**–**XI**

analogue		HPLC t_r (min)	formula	$[M + H^+]$ (m/z)		yield[a] (%)	
				calculated	found	A	B
[Mpa[1],Acc[2]]AVP	**I**	9.47	$C_{44}H_{66}N_{14}O_{11}S_2$	1031.2	1031.5	30	29
[Acc[2],Val[4]]AVP	**II**	8.91	$C_{44}H_{67}N_{14}O_{10}S_2$	1016.2	1017.7	77	21
[Cpa[1],Acc[2],Val[4]]AVP	**III**	13.88	$C_{49}H_{75}N_{13}O_{10}S_2$	1070.3	1070.7	83	65
[Mpa[1],Acc[2],D-Arg[8]]VP	**IV**	9.35	$C_{44}H_{66}N_{14}O_{11}S_2$	1031.2	1031.5	39	17
[Cpa[1],Acc[2],D-Arg[8]]VP	**V**	11.95	$C_{49}H_{74}N_{14}O_{11}S_2$	1099.3	1099.5	85	61
[Mpa[1],Acc[2],Val[4],D-Arg[8]]VP	**VI**	11.66	$C_{44}H_{64}N_{13}O_{10}S_2$	999.7	1001.2	80	45
[Acc[3],Val[4]]AVP	**VII**	6.95	$C_{44}H_{68}N_{14}O_{11}S_2$	1033.2	1033.1	77	43
[Cpa[1],Acc[3],Val[4]]AVP	**VIII**	10.73	$C_{49}H_{75}N_{13}O_{11}S_2$	1086.3	1086.8	87	81
[Mpa[1],Acc[3],D-Arg[8]]VP	**IX**	7.11	$C_{44}H_{66}N_{14}O_{12}S_2$	1047.2	1047.5	33	11
[Cpa[1],Acc[3],D-Arg[8]]VP	**X**	9.17	$C_{49}H_{74}N_{14}O_{12}S_2$	1115.3	1115.2	92	73
[Mpa[1],Acc[3],Val[4],D-Arg[8]]VP	**XI**	8.75	$C_{44}H_{67}N_{13}O_{11}S_2$	1018.2	1018.2	52	45

[a] A: yields were calculated on the base of the glycine content of the starting resin. B: yields are based on the amount of crude peptide.

Table 2. Pharmacological Properties of New Analogues of AVP

analogue		activity		
		uterotonic in vitro, no Mg^{2+} (IU/mg or pA_2)	pressor (IU/mg or pA_2)	antidiuretic[b] (IU/mg)
AVP[a]		17	412	465
[Val[4]]AVP			32	738
[D-Arg[8]]VP[a]		0.4	4.1	114–257
[Mpa[1]]AVP[a]		27–63	346–370	1300–1745
[Cpa[1]]AVP[a]		pA_2 = 8.15	pA_2 = 8.35	0.033
[Mpa[1],D-Arg[8]]VP[a]		1.5–5.1	~0.39	800–50000
[Acc[2]]AVP[c]		pA_2 ≈ 5.6	56.6	750–900 (~9300)
[Acc[2],D-Arg[8]]VP[c]		pA_2 ≈ 5.7	pA_2 ≈ 5.8	750–900 (~9300)
[Cpa[1],Acc[2]]AVP[c]		pA_2 = 7.33	pA_2 = 7.26	~0.1 (~1.4)
[Acc[3]]AVP[c]		0	0.24	~0.1 (~1.4)
[Mpa[1], Acc[3]]AVP[c]		<0.04	0.34	~0.8 (~25)
[Acc[3],D-Arg[8]]VP[c]		0	0	~0.1 (~1.4)
[Cpa[1], Acc[3]]AVP[c]		0	0	~0.8 (~25)
[Mpa[1],Acc[2]]AVP	**I**	pA_2 = 6.1 and 0.7 IU/mg	17.2 ± 0.8	~4500 (50000)
[Acc[2],Val[4]]AVP	**II**	pA_2 = 6.9	0.9 ± 0.2	~2300 (23000)
[Cpa[1],Acc[2],Val[4]]AVP	**III**	pA_2 = 6.96	pA_2 = 6.87	<0.4
[Mpa[1],Acc[2],D-Arg[8]]VP	**IV**	pA_2 = 6.5 and 0.3 IU/mg	pA_2 = 5.70	~4500 (50000)
[Cpa[1],Acc[2],D-Arg[8]]VP	**V**	pA_2 = 7.32	pA_2 = 6.63	<4
[Mpa[1],Acc[2],Val[4],D-Arg[8]]VP	**VI**	pA_2 = 7.81	pA_2 = 6.14	~4500 (50000)
[Acc[3],Val[4]]AVP	**VII**	0	0	<0.4
[Cpa[1],Acc[3],Val[4]]AVP	**VIII**	pA_2 ≤ 6.0	0	<4
[Mpa[1],Acc[3],D-Arg[8]]VP	**IX**	0	0	<4
[Cpa[1],Acc[3],D-Arg[8]]VP	**X**	0	0	<0.4
[Mpa[1],Acc[3],Val[4],D-Arg[8]]VP	**XI**	0	0	450 (4500)

[a] Values taken from ref 3. [b] The activities were obtained by comparing doses of AVP and the analogue resulting in an antidiuresis time of $t_{1/2}$ = 60 min. In parentheses, the activities were obtained by comparing doses of AVP and the analogue resulting in an antidiuresis time of $t_{1/2}$ = 200 min. [c] Values taken from ref 12.

Acc[2],Val[4]]AVP (**III**), [Mpa[1],Acc[2],D-Arg[8]]VP (**IV**), [Cpa[1],-Acc[2],D-Arg[8]]VP (**V**), [Mpa[1],Acc[2],Val[4],D-Arg[8]]VP (**VI**), [Acc[3],Val[4]]AVP (**VII**), [Cpa[1],Acc[3],Val[4]]AVP (**VIII**), [Mpa[1],-Acc[3],D-Arg[8]]VP (**IX**), [Cpa[1],Acc[3],D-Arg[8]]VP (**X**), and [Mpa[1],Acc[3],Val[4],D-Arg[8]]VP (**XI**). The proposed modifications should also enhance the resistance of the resulting peptides to enzymic degradation.

Results

A total number of 11 new analogues of AVP (**I**–**XI**) were synthesized by Fmoc or Boc strategy and characterized. Their physicochemical properties are presented in Table 1. The values of the molecular ions were as expected.

Results of pharmacological evaluation of the new analogues **I**–**XI**, together with relevant values for AVP and some related peptides, are presented in Table 2. The activities of the analogues were determined in the in vitro rat uterotonic test in the absence of magnesium ions, the rat pressor test, and the antidiuretic assay on conscious rats as described in Experimental Section.

It is difficult to compare the data of the antidiuretic activity of different analogues because the published values were obtained using different procedures. In the test using conscious rats, the slopes of the dose–response curves for some compounds (e.g., [Mpa[1]]AVP or [Mpa[1],D-Arg[8]]VP) are much steeper than that of vasopressin. This means that the same increase in the applied dose of either of the two compounds produces a much greater increase in activity than in the case of vasopressin. The slope of the dose–response curves of analogues **I**, **II, IV**, **VI**, and **XI** is also steeper than that of AVP, as was the case with Acc analogues described in ref 12. Thus, to compare the effects of the modifications with the parent compounds, we again compared such doses of AVP and new analogues that gave the same antidiuretic response, i.e., the doses that caused the time in which the rats excreted half of the water load ($t_{1/2}$) to be 60 and 200 min. For AVP the activity

was calculated to be about 465 IU/mg. None of the compounds exhibited diuretic, or in other words anti-antidiuretic, activity.

Peptides modified in position 2 with Acc exhibited weak (**I**, **II**, and **IV**), moderate (**III** and **V**), or strong **(VI)** antiuterotonic activities. Two analogues (**I** and **II**) showed moderate or weak pressor agonism, whereas compounds **III**−**VI** were moderately or weakly potent pressor antagonists. With respect to antidiuretic activity, peptides **I**, **II**, **IV**, and **VI** turned out to be highly potent agonists, while analogues **III** and **V** exhibited only weak agonistic properties.

Upon moving to analogues substituted with Acc in position 3, it is clear that this modification was deleterious for all the activities studied because only peptide **XI** was a relatively potent and very selective V_2 agonist. The remaining peptides showed only very weak antidiuretic activity and practically did not interact with the V_1 or oxytocin receptors (with the exception of **VIII**, which exhibited very weak antiuterotonic properties).

Discussion

The present work is a continuation of our studies aimed at clarifying the impact of sterical restrictions in the N-terminal part of the AVP molecule on pharmacological properties. Previously we reported that the bulky naphthyl moiety in position 2 or 3 of AVP and some of its analogues influenced significantly the interaction with V_{1a}, V_2, and oxytocic receptors as expressed by modulated pharmacological activities.[10,14] Some of the peptides displayed very interesting pharmacological properties. One of these new compounds, [L-2-Nal^3,D-Arg^8]VP, was the first potent V_{1a} antagonist devoid of antiuterotonic activity. In other studies, we imposed steric restrictions by replacement of amino acid residues in positions 2 and 3 with *N*,*N*′-ethylene-bridged dipeptide Phe−Phe. Again, this resulted in highly potent and selective V_{1a} antagonists.[15] Recently, we substituted Acc in position 2 or 3 of AVP and some of its analogues to reduce flexibility of peptide chains. The Acc modification, apart from reducing the flexibility, also changed the character of a fragment of the molecule from aromatic to aliphatic. As mentioned in the Introduction, Acc^3 modification was deleterious for interaction of the analogues with V_{1a}, V_2, and oxytocic receptors, whereas Acc^2 substitution selectively modulated the interaction with the receptors.

The results presented in Table 2 show that the combination of Acc^2 substitution with either Val^4 (analogue **II**) or Mpa^1 (analogue **I**) modification greatly increases the antidiuretic potency of the resulting peptides, compared to parent compounds [Acc^2]AVP, [Mpa^1]AVP and [Val^4]AVP. The enhancement of activity is, in the case of [Mpa^1,Acc^2]AVP, about 2 times higher than in the case of [Acc^2,Val^4]AVP, this being compatible with previous knowledge.[16,17] In comparison to [Mpa^1]-AVP and [Val^4]AVP, the Acc substitution in position 2 improves selectivity of the antidiuretic effect.

Moving a step further, we decided to synthesize an analogue in which we combined Acc^2 and Mpa^1 substitutions with inversion of configuration of the Arg^8 residue. The D-Arg^8 modification has commonly been used to improve peptide selectivity.[1,2,17] Antidiuretic and antiuterotonic activities of the resulting analogue **IV** did not change, whereas its pressor properties converted from weak agonistic to weak antagonistic (compared with analogue **I**), which improved selectivity. Finally, combination of Acc^2 substitution with the three above-mentioned modifications resulted in analogue **VI** of antidiuretic activity matching that of compounds **I** and **IV** but of significantly higher antipressor and antiuterotonic potency.

The introduction of D-Arg^8 or Val^4 modifications into the [Cpa^1,Acc^2]AVP molecule (analogues **III** and **V)** did not significantly change the potencies compared with the parent compound.

As far as the analogues obtained by substitution of Phe in position 3 with Acc are concerned, this modification was disadvantageous for all the activities studied. However, in this context, it is interesting to note a relatively high, similar to that of AVP, antidiuretic activity of peptide **XI**. It is also worth emphasizing that this analogue is exceptionally selective because it did not show any pressor or uterotonic activities up to a dose of 0.2 mg/kg or 5×10^{-6} M concentration, respectively. Moreover, it should be pointed out that this peptide differs from peptide **IX**, which is practically inactive, only in the presence of Val in position 4. Also, compounds in which we combined Acc^3 modification with other substitutions proposed (**VII**, **VIII**, **X**) did not show any significant activity. Previously, we demonstrated that it was also the case with D-Arg^8 substitution.[12]

Conclusion

Our studies resulted in four highly potent V_2 agonists (**I**, **II**, **IV**, and **VI**) with different uterotonic and pressor activities and one moderately potent but selective V_2 agonist (**XI**). These compounds may serve as extremely useful pharmacological probes for further evaluation of the role of AVP in the regulation of water excretion. Our findings demonstrated once more that limitation of conformational freedom by incorporation of conformationally restricted amino acid residues into the N-terminal part of AVP could result in peptides with useful pharmacological properties. Moreover, the exceptionally high antidiuretic potency of analogues **I**−**IV** supports our earlier hypothesis[12] that an amino acid residue in position 2 has a significant impact on this activity. In view of our present and previous results[12,15,16] and bearing in mind that some of them were rather surprising and poorly compatible with previous knowledge, we suggest that these analogues should be investigated for changes in the three-dimensional shape of the molecules, using nuclear magnetic resonance and theoretical methods. These useful tools may contribute to the explanation of closer connections.

In summary, our studies provide useful information about structure−activity relationships and open up new possibilities for the design of very potent and selective V_2 agonists.

Experimental Section

Thin-layer chromatography (TLC) was carried out on silica plates (Merck), and spots were visualized with iodine or ninhydrin. The solvent system used was 1-butanol/acetic acid/water/ethyl acetate (1:1:1:1, v/v). High-performance liquid chromatography (HPLC) was carried out on a Waters (analytical and preparative) chromatograph equipped with a UV detector (λ = 226 nm). The purity of the peptides was

determined on a Waters C_{18} column (5 μm, 100A; 150 mm × 3.9 mm). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA), [B] acetonitrile/0.1% aqueous TFA (80:20 v/v). Linear gradients from 20% to 80% of solution B was applied to peptides **I**−**VIII**, **X**, and **XI** and from 20% to 40% of solution B for **IX** for 20 min at a flow rate of 1 mL/min. Preparative HPLC was carried out using a Kromasil C_8 column (5 μm, 25 mm × 250 mm) in a gradient running from 10% to 50% of solution B for 120 min at a flow rate of 10 mL/min. FAB/MS of the peptides were recorded on a MALDI TOF mass spectrometer.

Mpa(Trt) was obtained as described for Cys(Trt)[18] using 3-mercaptopropionic acid instead of L-cysteine hydrochloride. Mpa(Mob) was obtained as described for Mpa(Bzl)[19] using *p*-methoxybenzyl chloride. Cpa(Mob) was synthesized using a procedure described in the literature.[20]

All amino acid derivatives were purchased from NovaBiochem except Boc-Acc and Fmoc-Acc, which were provided by Chem-Impex Int., Inc.

Peptide Synthesis. All peptides were obtained by solid-phase peptide synthesis. The syntheses of analogues **I**, **II**, **IV**, **VI**, **VII**, **IX**, and **XI** were performed on a Symphony synthesizer (Protein Technologies, Inc.) using Fmoc chemistry on polystyrene resin (Fmoc-Gly TentaGel S RAM, capacity 0.22 mmol/g) on a scale of 150 μmol, and peptides **III**, **V**, **VIII**, and **X** were synthesized manually using Boc chemistry on a methoxybenzhydryl resin (MBHA resin, Senn Chemicals AG, 1% DVB, 200−400 mesh, 0.67 mmol/g) on a scale of 200 μmol according to standard procedures, using in situ neutralization.[21] Analogues **VI** and **XI** synthesized using a Fmoc strategy were obtained in very low yields, and we resynthesized them by Boc chemistry.

Mixtures of protected amino acid/TBTU/HOBt/DIEA (1:1:1:2) in DMF or protected amino acid/HATU/HOAt/DIEA (1:1:1:2) in DMF or in a mixture of DMF/NMP (1:1 v/v) containing 1% Triton were used for coupling. The completeness of each coupling reaction during manual synthesis was monitored by the Kaiser test[22] or chloranil test.[23] Recoupling was performed when the test was positive. With peptides **I**, **III**−**VI**, and **VIII**−**XI**, Mpa(Trt), Mpa(Mob), or Cpa(Mob) were used in the final coupling step.

The Fmoc deprotection was accomplished using a 20% solution of piperidine in DMF. A solution of TFA/H_2O/TIS/PhOH (92.5:2.5:2.5:2.5) was used for the cleavage of peptides from the TentaGel resin (3 h). Solutions of the cleaved peptides were filtered off and evaporated in vacuo to ca. 1 mL. Then the peptides were precipitated with diethyl ether to afford crude products.

The Boc deprotection was accomplished using a 33% solution of TFA in DCM. The protected peptidyl or acylpeptidyl resins were treated with 10 mL of liquid hydrogen fluoride (HF) containing 1 mL of anisole at −70 °C and stirred for 60 min at 0 °C.[24] After removal of HF and anisole in vacuo, the mixture was washed successively with anhydrous diethyl ether and acetic acid and the solution was diluted with methanol.

The resulting dithiols were oxidatively cyclized with 0.1 M I_2 in methanol using the standard procedure. The solvents were evaporated under reduced pressure, and residue was dissolved in water and lyophilized. The crude products were desalted on a Sephadex G-15 column and eluted with aqueous acetic acid (30%) at a flow rate of 3 mL/h. After freeze-drying, the fractions comprising the major peak were purified by RP-HPLC. The peptides were eluted as single peaks. The purity and identity of each peptide was determined by HPLC and FAB mass spectrometry (molecular ion).

Biological Evaluation. Wistar rats were used in all experiments. Female rats were estrogenized 48 h before the experiment. The uterotonic test was carried out in vitro in the absence of magnesium ions.[25,26] The vasopressor test was performed using phenoxybenzamine-treated male rats.[27] Synthetic oxytocin was used as a standard in uterotonic tests, and synthetic arginine vasopressin was used in the pressor test. Dose−response (single administration) or cumulative dose−response (measurements without washing steps between the administration of enhanced doses) curves were constructed. The values reported are averages of three to five separate experiments.

Tests to assess the antidiuretic or diuretic properties were conducted on conscious male rats in two variations of the modified Burn test.[28,29] In the standard manner with hydrated rats, the animals having fasted for 16 h were weighed and then given tap water through a stomach catheter. The water load was 4% of the body weight. Immediately after the water load, the tested substances (or physiological saline as control) were administered subcutaneously at doses of 0.001−100 nmol/kg. The rats were then placed in individual metabolic cages, and their urine was collected over a 5 h period. The time $t_{1/2}$ in which the rats excreted half the water load was determined and then plotted against the dose. For comparison of the compounds, such doses were chosen to yield $t_{1/2}$ equal to 200 min and the so-called threshold doses yielding $t_{1/2}$ equal to 60 min (equal to the value of $t_{1/2}$ obtained with the physiological solution). On each day of the experiment, 21 rats divided into 5 groups of 4 or 5 animals to which different doses and compounds were administered were used; each dose being tested in two or three independent experiments (different days, different rats). To test for diuretic effects with nonhydrated rats, no water load was given to the fasting animals. For details see ref. 30.

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Appendix

Abbreviations. The symbols of the amino acids and peptides are in accordance with the 1983 Recommendations of the IUPAC−IUB Joint Commission on Biochemical Nomenclature and "A Revised Guide to Abbreviations in Peptide Science" published in *J. Pept. Sci.* **2003**, *9*, 1−8. Other abbreviations are the following: Acc, 1-aminocyclohexane-1-carboxylic acid; AVP, arginine vasopressin; Cpa, 1-mercaptocyclohexaneacetic acid; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; MBHA, *p*-methylbenzhydrylamine; Mob, 4-methoxybenzyl; Mpa, 3-mercaptopropionic acid; Nal, β-(1-naphthyl)-L-alanine; NMP, 1-methyl-2-pyrrolidone; OT, oxytocin, TBTU, 2-1H-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, trityl.

Supporting Information Available: HPLC retention times and purities of **I**−**XI**. This material is available free of charge via the Internet at http://pubs.acs.org.

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